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Integration and expression of a rabbit liver cytochrome P-450 gene in transgenic *Nicotiana tabacum*

(senescence/metabolism of nicotine/*Agrobacterium tumefaciens*/Ti plasmid vector/promoter for mannopine synthase)

KAZUKI SAITO*†, MASAAKI NOJI*, SHIGERU OHMORI*, YOSHIO IMAI‡, AND ISAMU MURAKOSHI*

*Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan; and ‡Faculty of Agriculture, Osaka Prefecture University, Sakai 591, Japan

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ABSTRACT **Cytochrome P-450 is involved in the oxidative metabolism of a broad range of substrates. We have made a chimeric construct, pSN002, containing the cDNA for rabbit liver cytochrome P-450 (IIC14) under the control of the TR2′ promoter for mannopine synthase in the *Agrobacterium* Ti plasmid. *Nicotiana tabacum* was transformed with *Agrobacterium tumefaciens* harboring a cointegrated plasmid pSN002::pGV2260. The presence of mRNA and of the translated protein from the chimeric cytochrome P-450 gene in transgenic plants was confirmed by RNA blot hybridization and by Western blot and immunohistochemical analyses, respectively. The transformants in which the foreign cytochrome P-450 protein is expressed show marked phenotypic changes, notably a tendency rapidly to senesce. We detected 2-propenylpyrrolidine, a degradative metabolite of nicotine alkaloids, in transgenic tobacco showing this pronounced phenotypic change. Such metabolism is likely to be due to the effect of senescence and not directly to the presence of the cytochrome P-450.**

The P-450 proteins are protoheme-containing monooxygenases and are widespread in nature. These enzymes are involved in the oxidative metabolism of a broad range of substrates (1). Mechanistic and structural studies have been performed largely using a preparation of bacterial (*Pseudomonas putida*) camphor hydroxylase (2). The P-450 enzymes in mammalian liver cells have been extensively studied because of their importance in xenobiotic and drug metabolism (3, 4). Knowledge of the genetic organization, expression, regulation, and catalytic properties of P-450 has been obtained using mammalian systems coupled with an expression system in yeast (1, 5, 6). Although in this way considerable progress has been made in characterizing bacterial and mammalian P-450 proteins, P-450 proteins in plants still remain poorly understood. In plants, the P-450 monooxygenases catalyze many oxidative reactions in the biosynthetic pathways for secondary products, in phytohormone metabolism, and in herbicide detoxification (7). At present, only one plant P-450 cDNA, from ripening avocado fruit, has been cloned and partially characterized (8).

We are currently studying the genetic manipulation of plant secondary metabolism by expressing foreign genes in transgenic plants (9–11). In this context, the mammalian P-450 is interesting for the following reasons. (*i*) In mammalian liver cells, some xenobiotics and drugs, including plant products, are oxidized to more highly bioactive metabolites by the action of P-450 (12). (*ii*) Toxic compounds are detoxified by oxidative reactions of P-450. (*iii*) The general broad-substrate-specificity electron transfer activity of P-450 in microsomal membrane (13) may initiate unpredictable metabolic changes concomitant with the expression of foreign P-450. This may, in turn, induce some phenotypic changes and subsequent changes in metabolite pattern of transgenic plants.

In the present study, we have obtained transgenic *Nicotiana tabacum* carrying an integrated chimeric cDNA for a rabbit liver P-450 by using *Agrobacterium*-mediated transformation. The transformants in which the P-450 protein is expressed show marked phenotypic changes, notably, a tendency to senesce rapidly and to accumulate degradative metabolites of nicotine alkaloids.

MATERIALS AND METHODS

Plasmids and Bacteria. Plasmid pUF3 is a derivative of pUC19 containing the entire cDNA coding sequence (pHP3) for a minor form of P-450 (IIC14) from phenobarbital-treated rabbit liver (14). The 1.6-kilobase (kb) *Bam*HI fragment of pUF3 contains a 1470-base-pair reading frame and 3′ flanking region of pHP3. This 1.6-kb fragment was excised and cloned into the dephosphorylated *Bam*HI site of the plant expression vector pGSH160 (15) in the correct orientation to give a chimeric intermediate vector pSN002 (Fig. 1), in which the cDNA is under the transcriptional control of the TR2′ promoter for mannopine synthase (16, 17). The nucleotide sequence at the junction between the TR2′ promoter and the coding sequence was determined by the dideoxynucleotide method (18). Plasmid pSN002 was mobilized into *Agrobacterium tumefaciens* harboring the disarmed Ti plasmid pGV2260 (19) by triparental mating using pRK2013 as a helper plasmid (20). The transconjugated *Agrobacterium* containing cointegrated pSN002::PGV2260 was selected on agar plates containing rifampicin (50 μg/ml) and spectinomycin (100 μg/ml) and checked by Southern blot hybridization of total DNA as described (21).

Plant Transformation. Leaves of a sterile shoot culture of *N. tabacum* cv. Petit Havana SR1 were used for transformation with *Agrobacterium*. Leaf-disc transformation and regeneration were carried out essentially as reported (22).

DNA and RNA Blot Hybridizations. Isolation of plant DNA and Southern blot analysis were performed as reported (10). The 1.6-kb *Bam*HI fragment and the 1.4-kb *Nco* I-*Hin*dIII fragment of pSN002 were used as ^{32}P-labeled probes.

Plant RNA was isolated from leaves as described (23). For RNA gel blots, 20 μg of total RNA was denatured and electrophoresed on a formaldehyde agarose (1.17%) gel, followed by transfer to an Immobilon-N membrane filter (Millipore). Prehybridization and hybridization were carried out in 5× SSPE/50% (vol/vol) formamide/5× Denhardt's solution/0.1% SDS/2% (wt/vol) dextran sulfate/denatured salmon sperm DNA (250 μg/ml) at 50°C using random-

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Abbreviations: GC/MS, gas chromatography-mass spectrometry; T-DNA, transferred DNA.

†To whom reprint requests should be addressed at: Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Chiba 260, Japan.

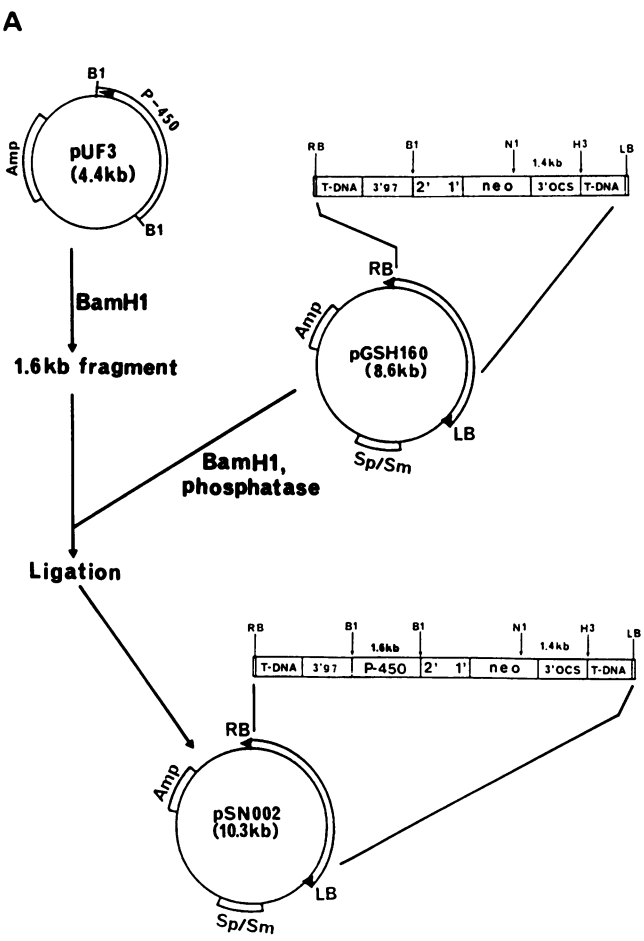

FIG. 1. (*A*) Construction of the intermediate vector pSN002. pUF3 containing the entire P-450 cDNA from pHP3 (14) was digested with *Bam*HI to give a 1.6-kb fragment. This fragment was isolated and ligated into the *Bam*HI-digested alkaline phosphatase-treated pGSH160 (15). The resulting expression vector pSN002 contained a chimeric P-450 gene under the control of the TR2′ promoter and flanked with the fragment for termination and polyadenylylation of the T-DNA gene 7 (3′g7). This plasmid also contained a chimeric gene for neomycin resistance as a selectable marker flanked with TR1′ promoter and the terminator of octopine synthase of T-DNA (3′OCS). RB, right border; LB, left border; Amp, ampicillin-resistance marker; Sp/Sm, streptomycin- and spectinomycin-resistance marker; B1, *Bam*HI; N1, *Nco* I; H3, *Hin*dIII; 2′ and 1′, promoters TR2′ and TR1′, respectively, for mannopine synthase of T_R-DNA (16, 17). (*B*) Nucleotide sequence of junction between TR2′ promoter and the P-450 cDNA (IIC14). The first ATG codon and the *Bam*HI site are indicated.

primer-labeled ^{32}P probes (Takara Shuzo, Kyoto) (1× SSPE = 0.18 M NaCl/0.01 M sodium phosphate, pH 7.7/1 mM Na_2EDTA). Filters were washed in 2× standard saline citrate (SSC) for 30 min at 50°C and then in 2× SSC/0.1% SDS for 10 min at 50°C. An RNA ladder (Bethesda Research Laboratories) was used for size markers.

Immunostaining. For Western blots, the microsomal protein was obtained from leaves as described (24). Western blot analysis on an Immobilon-P membrane filter (Millipore) with peroxidase staining was carried out essentially as reported (25) using 4-chloro-1-naphthol as developer (26). The primary antibody against a rat P-450 (IIC11) was prepared as described (27) and used at a 1:200 dilution in phosphate-buffered saline (PBS).

For immunohistochemical staining, fresh plant material was cut by hand with a blade. Thin transverse sections of stems were washed with PBS once and incubated in primary antibody (rabbit anti-P-450 IIC11) diluted 1:200 in PBS for 1 hr at 25°C. The sections were washed six times with PBS, incubated with the second antibody (goat anti-rabbit IgG) (Cappel Laboratories) for 15 min, and washed six times with PBS. Subsequently, a peroxidase-anti-peroxidase complex (rabbit) (BioMakor, Rehovot, Israel) in PBS was incubated for 15 min at 25°C and washed six times with PBS. The sections were developed in PBS containing 0.8 mM 3,3′-diaminobenzidine and 1.4 mM hydrogen peroxide.

Detection of Alkaloids. The total alkaloidal fraction was obtained from leaves of *N. tabacum* and analyzed by gas chromatography/mass spectrometry (GC/MS) as described (9).

Miscellaneous Techniques. General recombinant DNA techniques were as in Maniatis *et al.* (28) in accordance with the *Guidelines for Research Involving Recombinant DNA Molecules* issued by the Ministry of Education, Science and Culture of Japan. Protein was determined by the method of Bradford (29). The neomycin phosphotransferase assay was performed as described (30). The content of chlorophyll in the leaves was determined fluorophotometrically as described (11). The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde content by the thiobarbituric acid reaction (31).

RESULTS

Construction of the Chimeric P-450 Gene and Plant Transformation. The cDNA of a rabbit P-450 (IIC14) isolated from phenobarbital-treated liver cells (14) was cloned into the intermediate vector pSN002 flanked by the TR2′ promoter and the 3′ end of the transcript 7 of the Ti plasmid (Fig. 1). The specific expression of the TR2′ promoter in phloem of tobacco has been shown (32, 33). Plasmid pSN002, a derivative of pGSH160 (15), also contained a chimeric neomycin-resistance gene as a selectable marker for kanamycin resistance in transgenic plant cells.

Transgenic plants were obtained 6–7 months after *Agrobacterium* infection. That it took longer than normal (3–4 months) to obtain transgenic tobacco indicates that the transferred genes may have been having some metabolic effects. Southern blot hybridization of total DNA from transgenic tobacco, using the 1.6-kb *Bam*HI fragment as probe,

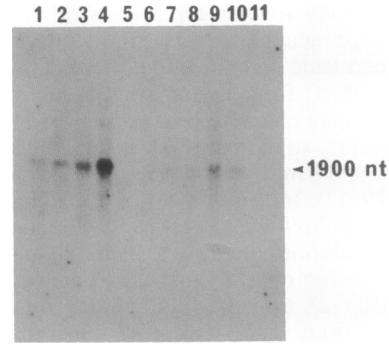

FIG. 2. Northern blot hybridization of tobacco leaf tissues. Denatured total RNA (20 μg) from leaves was electrophoresed on an agarose gel (1.17%), transferred to an Immobilon-N filter, and hybridized with the ^{32}P-labeled 1.6-kb *Bam*HI fragment of pSN002 as a probe. nt, nucleotides. The following transformants were used. Lanes: 1, transformant 103; 2, 108; 3, 112; 4, 113; 5, 116; 6, 118; 7, 119; 8, 121; 9, 122; 10, 123; 11, nontransformed control.

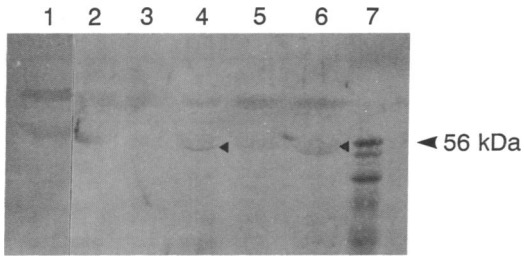

FIG. 3. Western blot analysis of the microsomal protein of the leaves of the transformants. The protein was electrophoresed on an SDS/polyacrylamide gel, transferred onto an Immobilon-P filter, and localized by immunostaining. Lanes: 1, SR1, nontransformed control; 7, yeast, microsomal protein of yeast harboring the expression vector pAHF3 derived from pHP3. Lanes containing various transformants are as follows. Lanes: 2, 121; 3, 119; 4, 113; 5, 112; 6, 108. Note: The expressed P-450 protein migrates slightly faster than a prominent protein of the microsomes around 56 kDa and is detected in transformants 113 and 108 (indicated by arrowheads). In the yeast extract, the immunoreacting proteins of lower molecular mass than the intact 55.7-kDa protein are polypeptide fragments derived from the intact P-450 protein by proteolysis during the extraction procedure.

showed that one to three transferred DNA (T-DNA) copies were integrated per genome (data not shown). Normal levels of neomycin phosphotransferase activity in the transformants were observed by *in situ* assay on native polyacrylamide gels (30).

Expression of a Foreign P-450 Gene. The presence of mRNA due to the expression of the foreign DNA encoding for the rabbit liver P-450 was confirmed by Northern blot hybridization (Fig. 2). The level of expression varied for individual transformants, possibly because of positional effects of T-DNA insertion and the different number of gene copies. However, the length of the transcript was ≈1900 nucleotides, as expected with the addition of a poly(A) tail. Transformant 113 gave the highest level of mRNA expression, followed by transformants 112 and 108.

Western blot analysis with peroxidase staining was carried out using an antibody raised against a rat P-450 (IIC11), which is in the same subfamily as the integrated P-450 (IIC14) and with which it cross-reacts (Fig. 3). Low but significant amounts of P-450 protein were detected in the microsomal fraction of transformants 113 and 108, but not in other transformants. The P-450 protein with a predicted size of 55.7 kDa migrated just in front of a prominent ≈56-kDa polypeptide band from the microsomal fraction of tobacco. The control serum raised against a rat P-450 IIB1 gave no detectable reaction product around 56 kDa.

Immunohistochemical staining of transverse sections of stems, using the same antibody, indicated that the P-450 protein was present in phloem of transformant 113, in particular in internal phloem, but not in a nontransformed control (Fig. 4). This phloem-specific expression of the TR2′ promoter is the same pattern observed with a chimeric construct of the TR2′ promoter and β-glucuronidase (32, 33). The β-glucuronidase staining was also primarily evident in the internal phloem (33).

Changes in Phenotype and Metabolite Accumulation. Marked phenotypic change was observed in transformants 113, 108, and 117 (Fig. 5). These transformants had a tendency rapidly to senesce and flower compared with nontransformed controls. Senescence has been characterized by a breakdown of chlorophyll and increased levels of lipid peroxidation (31, 34). Therefore, the amounts of chlorophyll and malondialdehyde, a degradative product of lipid peroxidation, were determined (Table 1). The tendency for rapid senescence in transformants 113 and 108 was clearly indicated by the low levels of chlorophyll and the high concentrations of malondialdehyde compared with those in the control. Transformant 117, confirmed to be transgenic by Southern blot analysis, had the most pronounced phenotypic changes among the transformants obtained, but the plant was made too fragile by senescence to be subjected to further analysis.

When the alkaloidal fractions of the leaves of transformants 117, 113, and 108 were analyzed by GC/MS (Fig. 6), an alkaloid with a molecular ion at m/z 111 was detected in addition to the three normal tobacco alkaloids (e.g., nicotine,

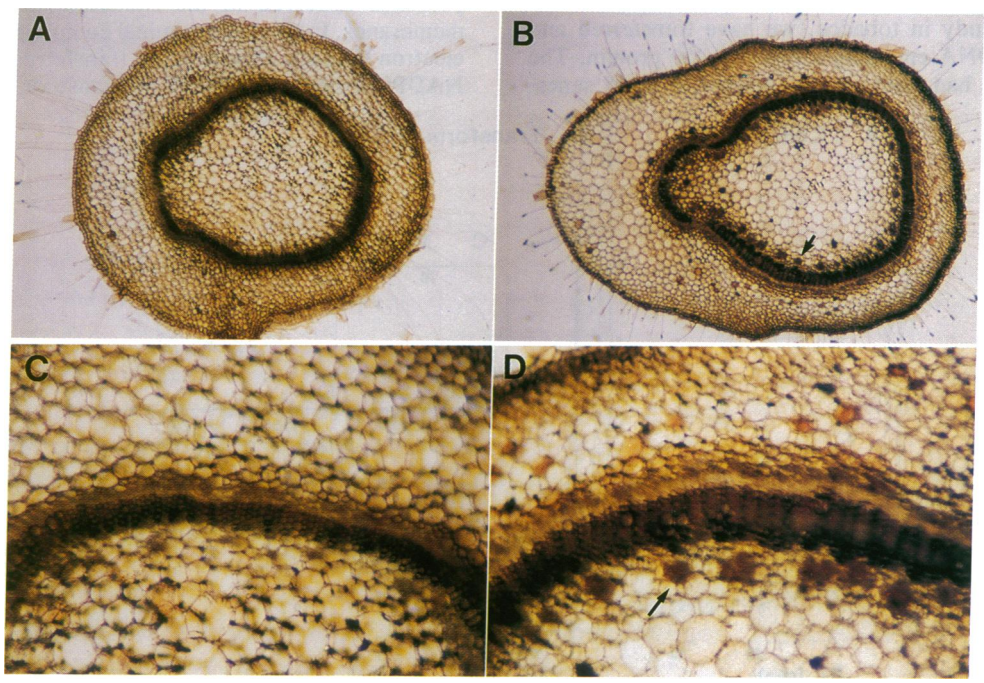

FIG. 4. Immunohistochemical localization of P-450 protein. Transverse sections of stem of the transformant 113 and the nontransformed control were stained by using the antibody-peroxidase reaction and 3,3′-diaminobenzidine. (*A*) Nontransformed control. (×12.) (*B*) Transformant 113. (×12.) (*C*) Enlarged sector of nontransformed control. (×45.) (*D*) Enlarged sector of transformant 113. (×45.) Arrow, internal phloem.

FIG. 5. Phenotype of tobacco plants. The transformed tobacco 113 (*Right*) is compared with a nontransformed SR1 plant (*Left*). The plants were cultivated in A1 medium (22) at 25°C under illumination (2500 lux, 16 hr/day).

nornicotine, and anatabine). This alkaloid was predicted to be 2-propenylpyrrolidine based on a computer search of the Environmental Protection Agency/National Institutes of Health data base (35). This compound was also detected in highly senescent nontransformed tobacco but was absent in normal and healthy leaves. Thus, the accumulation of this compound is apparently an indirect consequence of heavy senescence in transgenic plants. The fact that this compound is certainly one of degradative metabolites of nornicotine also supports this prediction. The amount of nicotine was decreased in the transformant compared with that in the control. This is also a consequence of a senescence process (36).

DISCUSSION

In the present study in tobacco, we have transferred and expressed the cDNA encoding a rabbit P-450 protein. The transgenic plants had unexpected features of rapid senescence and rapid flowering. Furthermore, slow growth and rooting were observed in the transgenic plants containing the P-450 relative to the normal transformants. In parallel with this study, we have also obtained transgenic tobacco integrated with almost the same chimeric gene, the TR2′ promoter–*uidA*–3′ end of gene 7 (pGSGluc1), in which the P-450 cDNA is replaced with *uidA*, which encodes β-glucuronidase. In this construct no phenotypic changes were observed and we obtained transgenic plants within 4 months. However, for the P-450 construct, it took more than 6 months to obtain transformants. In addition a longer time (10–20 days) was required for rooting, after transferring the shoot to new medium, than for normal transformants (3–4 days).

Table 1. Concentrations of chlorophyll and malondialdehyde in *N. tabacum* plants

Sample	Chlorophyll, μg/g (fresh weight)	Malondialdehyde, nmol/g (fresh weight)
Nontransformed control SR1	786	7
Transformant 108	583	28
Transformant 113	168	13

Leaves of tobacco cultured for 3 months in A1 medium (22) were taken and the concentrations of chlorophyll (11) and malondialdehyde (31) were determined. Data are the mean of duplicate determinations.

Relatively low levels of P-450 protein were seen by Western blot analysis even in transformants that expressed the most mRNA (113 and 108). It is apparent, however, that the P-450 protein is located in the phloem at a high concentration. That higher levels of the P-450 protein accumulation are not observed may indicate that in transformants 108, 113, and 117 the level of accumulation of the P-450 protein is near the maximum tolerable; greater levels are probably lethal. A similar explanation for relatively low levels of a metabolically active protein being found in transgenic tobacco has been put forward to explain the low expression of a yeast ornithine decarboxylase (37).

There are several possible explanations for the rapid senescence observed in the transformants. In microsomal membranes, P-450 is the terminal component for microsomal electron transfer. Electrons are transferred from NADPH to NADPH–cytochrome P-450 reductase or cytochrome b_5 to

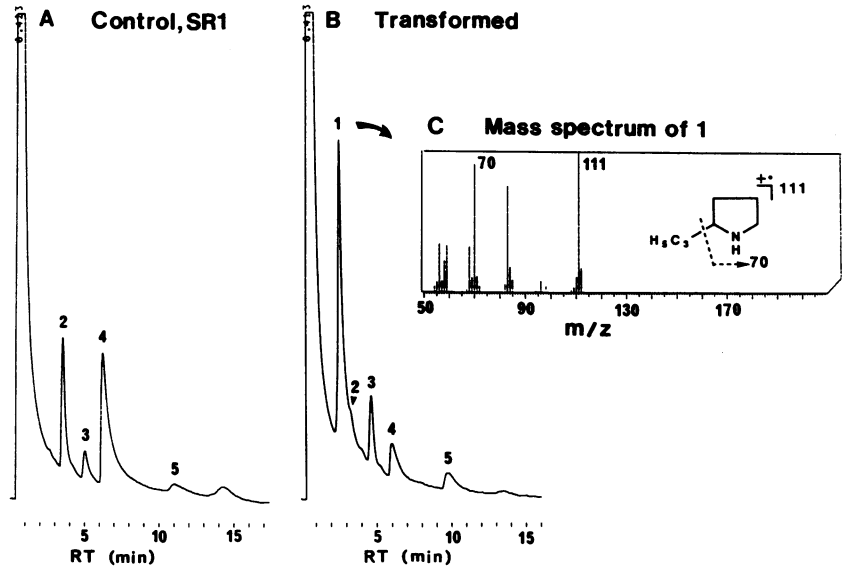

FIG. 6. GC/MS analysis of alkaloidal fraction from leaves of tobacco. The alkaloidal fractions from a nontransformed control SR1 plant (*A*) and transformant 117 (*B*) were analyzed by GC/MS. (*C*) Electron-impact mass spectrum of peak 1. Peaks: 1, 2-propenylpyrrolidine; 2, nicotine; 3, 4-methylquinoline (internal standard); 4, nornicotine; 5, anatabine. RT, retention time.

the terminal P-450, where they are used to oxidize substrates. It is possible that in those transgenic plants, in which the proper substrates are unavailable, electrons are transferred to surrounding unsaturated fatty acids causing lipid peroxidation (38) and subsequent senescence (31, 34). In support of this mechanism, Langrimini *et al.* (39) reported the wilting of transgenic tobacco carrying the peroxidase cDNA. This present study also indicates that nonregulated expression of a nonspecific enzyme for oxygen activation causes unfavorable wilting of plants. Another possibility involves the specific catalytic action of this P-450 (IIC14) enzyme to hydroxylate steroids. This P-450 can catalyze the 16 α-hydroxylation of testosterone (40). Thus, the membrane phytosterols or the phytohormonal brassinosteroids could be metabolized by the action of the foreign P-450. Such action will cause changes in membrane structure or in the phytohormone balance, either or both of which might cause the phenotypic changes observed. A further possibility is that perturbation in the structure and function of the microsomal membrane is caused by the introduction of numerous foreign hydrophobic P-450 molecules. Less directly, overexpression of this integrated foreign genes may lead to suppressive effects on related endogenous P-450 genes. Suppressive effects were reported (41, 42) for a transferred chalcone synthase gene. However, the underlying mechanism is still unclear.

The present study demonstrates that the integration of a foreign metabolically active gene into transgenic tobacco plants can have major metabolic consequences. In this instance, the gene is for a general P-450 oxygenase and has led to substantial phenotypic changes in the transformants. Although it is apparent that in some cases, such as glutamine synthase (43), a high expression can be obtained without any detrimental effects, it is clear that this is not always the case and that indirect consequences, such as the senescence seen in the present study or the cosuppression reported with chalcone synthase (41, 42), may also occur. These consequences of transgenic plant production may have serious repercussions in experiments designed to alter secondary metabolism by genetic techniques and indicate that the use of specific genes of plant origin, rather than genes encoding nonspecific activities as used here, may be advantageous.

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